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Sequence of occurring damages in yeast plasma membrane during dehydration and rehydration: Mechanisms of cell death

Hélène Simonin, Laurent Beney, Patrick Gervais *

Laboratoire de Génie des Procédés Alimentaires et Biotechnologiques ENSBANA-1, Esplanade Erasme-21000 DIJON, France

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Abstract

Yeasts are often exposed to variations in osmotic pressure in their natural environments or in their substrates when used in fermentation industries. Such changes may lead to cell death or activity loss. Although the involvement of the plasma membrane is strongly suspected, the mechanism remains unclear. Here, the integrity and functionality of the yeast plasma membrane at different levels of dehydration and rehydration during an osmotic treatment were assessed using various fluorescent dyes. Flow cytometry and confocal microscopy of cells stained with oxonol, propidium iodide, and lucifer yellow were used to study changes in membrane polarization, permeabilization, and endocytosis, respectively. Cell volume contraction, reversible depolarization, permeabilization, and endovesicle formation were successively observed with increasing levels of osmotic pressure during dehydration. The maximum survival rate was also detected at a specific rehydration level, of 20 MPa, above which cells were strongly permeabilized. Thus, we show that the two steps of an osmotic treatment, dehydration and rehydration, are both involved in the induction of cell death. Permeabilization of the plasma membranes is the critical event related to cell death. It may result from lipidic phase transitions in the membrane and from variations in the area-to-volume ratio during the osmotic treatment.

Keywords: Plasma membrane; Osmotic stress; *Saccharomyces cerevisiae*; Flow cytometry; Confocal microscopy

1. Introduction

In their natural habitats, plant surfaces, yeasts are exposed to a highly variable environment with respect to the availability of nutrients, temperature, and access to oxygen. Osmotic pressure, in particular, can vary widely and rapidly [1]. Yeasts may also be subjected to important osmotic pressure variations during technological processes. Thus, our understanding of the mechanism of cell death due to osmotic pressure variations in the environment is of fundamental interest. The viability rate after osmotic treatments depends on both the dehydration and the rehydration steps [2]. In fact, a slow increase in osmotic pressure and the application of an appropriate temperature during dehydration has been shown to preserve yeast viability [3,4]. Optimal osmotic pressure variation kinetics and temperatures for rehydration have also been demonstrated [5,6]. However, the mechanisms involved in these optimizations are not well understood.

The immediate effect of exposing *Saccharomyces cerevisiae* cells to hyperosmotic shock is a fast increase of osmotic pressure in the cell interior. It results from a rapid outflow of water and a slower entry of permeant solutes into the cell interior until a stationary state is reached, i.e. when extracellular and intracellular osmotic pressures are equal [7]. As a result, the volume of the cell decreases within seconds [8]. This passive osmotic response of cells involves the dehydration of the cell components and the shrinkage of the plasma membrane [9]. In the same way, a return to the initial osmotic pressure induces a cell volume increase [10,11] and rehydration of the cell components. As a consequence, the integrity and function of the plasma membrane are affected by osmotic pressure variations in the cellular environment.

The cytoplasmic membrane is a chemiosmotic barrier that provides the interface between the organism and its external

* Corresponding author. Tel.: +33 3 80 39 66 59; fax: +33 3 80 39 68 98.
E-mail address: gervais@u-bourgogne.fr (P. Gervais).

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environment. A transmembrane electrochemical potential (negative inside) across this phospholipid bilayer plays a pivotal role in the control of solute exchange. Increased plasma membrane permeability during dehydration or rehydration has been proposed as the main cause of cell death. In fact, increases and decreases in osmotic pressure induce the leakage of nucleotides, ions, and other soluble cell components into the surrounding medium [12–14].

The highly dynamic lipid bilayer of the plasma membrane is known to undergo phase transitions during dehydration [15] and rehydration [16,17]. On the one hand, the occurrence of a phase separation state resulting from the phase transition of some phospholipids in the membrane may be the cause of membrane rupture or changes in permeability, as proposed by Laroche and Gervais [18,19]. On the other hand, some authors suggest that the formation of endovesicles during dehydration leads to plasma membrane lysis during osmotic expansion at rehydration [2,20].

This study aimed to identify the sequence of events occurring in membranes that lead to cell death during dehydration and rehydration. To this end, the two phases of an osmotic treatment were considered, and the state of the plasma membrane was studied under hyperosmotic pressure at different levels of dehydration and rehydration. Osmotic treatments were carried out in glycerol solutions at osmotic pressures ranging from 1.4 to 144.4 MPa. Glycerol was chosen because it is a kosmotrope [21] meaning that it is preferentially solubilized within the bulk of the solution and excluded from the solvation layer of macromolecule surfaces. Thus, the main effect of increase in glycerol concentration is the decrease in water availability to hydrate macromolecules. Moreover this solute permits to prepare solutions at high osmotic pressures allowing cell staining with fluorescent dyes and their analysis. Three fluorescent dyes were used to assess plasma membrane integrity and functionality. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bisoxonol, BOX) is the reagent of choice for measuring changes in membrane potential [22]. It enters depolarized cells, where it binds to intracellular proteins and displays enhanced fluorescence. Propidium iodide (PI) stains nucleic acids and has been extensively used to detect dead or dying cells, which allow the dye to penetrate to the cell interior after the permeabilization of their plasma membranes [23]. Lucifer yellow (LY) is a membrane-impermeant anionic dye, which has been used to characterize endocytosis in plant cells [24] and yeasts [25]. Cells were studied individually with flow cytometry and confocal microscopy. These techniques allowed us to distinguish the membrane states of various subpopulations of cells by using a combination of different fluorescent dyes. BOX and PI were used in association for flow cytometric analysis to assess the functionality and integrity of cell membranes, as already reported [14,26]. LY was also used with PI to investigate the occurrence of a hypothetical endocytotic vesiculation phenomenon during osmotic treatments. These results were compared with cell viability, measured with the Colony Forming Unit (CFU) method after rehydration from hyperosmotic shocks of various magnitudes. Our results suggest that two mechanisms are involved in cell death during osmotic treatments: the non-lethal formation of endocytic vesicles during the dehydration step, leading to cell bursting at the later step of rehydration; and a lethal plasma membrane permeabilization during dehydration.

2. Materials and methods

2.1. Yeast suspension preparation

S. cerevisiae strain ATCC60218 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown aerobically at 30 °C in 250 mL conical flasks containing 100 mL of YM broth medium. A subculture (1 mL) was transferred into a conical flask containing the same medium, and cultures were placed on a rotary shaker (New Brunswick Scientific, Edison, NW, USA) at 250 rpm and allowed to grow to early stationary phase (48 h, final population of 1×10^8 cells mL^{-1}).

2.2. Preparation of binary water/glycerol solutions of different osmotic pressures

The Norrish equation [27] was used to determine the mass of solute to be added to 1000 g of distilled water to adjust the osmotic pressure to the desired level (Π):

$$\Pi = (1 - X_s)e^{-KXs^2}$$

where X_s is the molar fraction of the solute and K is the Norrish coefficient of the solute used to increase the osmotic pressure.

The solute used in all these experiments was glycerol (Sigma-Aldrich, Saint Quentin Fallavier, France), for which $K=1.16$ [28].

For fluorescence analysis, spectrophotometric-grade glycerol (Sigma-Aldrich) and phosphate buffer (10 mM, pH 5.8) were used to prepare the hyperosmotic solutions.

The osmotic pressure of all solutions was checked with a dew-point osmometer (Decagon Devices Inc., USA).

2.3. Measurement of cell volume

Samples (25 mL) of culture were centrifuged (5 min, 2200×*g*) and washed twice in a binary water/glycerol mixture (1.4 MPa). The pellets were resuspended in 2.5 mL of the same medium. An osmotic shock was induced by suddenly introducing 100 μL of cell suspension into 2.9 mL of binary water/glycerol solution (at final osmotic pressures of 1.4, 14.5, 30.7, 49.1, 70.3, 95.4, and 109.9 MPa). An aliquot (10 μL) of this final cell suspension was deposited onto a glass slide and the cells were examined with a Leica DMLB microscope (Leica Microsystèmes, Reuil-Malmaison, France). Cell volume variations were determined with an image analysis system attached to the microscope. Cells were individually analysed to determine their projected areas assuming that the cells were spherical as described previously [8].

2.4. Osmotic treatments

Samples (50 mL) of culture were centrifuged (5 min, 2200×*g*) and washed twice in a binary water/glycerol mixture (1.4 MPa). The pellets were resuspended in 5 mL of the same medium. Cell suspensions, rehydration solutions, and shock solutions were incubated for 10 min in a water bath placed in a thermostatically controlled room, to reach the appropriate temperature of 25 °C. The temperature was checked using a thermocouple. When the temperature was homogeneous, an osmotic shock was induced by suddenly introducing 1 mL of cell suspension into 9 mL of a binary water/glycerol solution (final osmotic pressures were 144.5, 109.9, 95.4, 70.3, and 39.6 MPa). The cells were maintained for 30 min under hyperosmotic conditions. Then 1 mL of the cell suspension in the shock solution was suddenly introduced into 9 mL of distilled water for rehydration.

For the flow cytometric assays, spectrophotometric-grade glycerol was dissolved in phosphate buffer (10 mM, pH 5.8) to prepare the hyperosmotic

solutions. For osmotic treatment at 144.5 MPa, eight levels of rehydration at decreasing osmotic pressures (72.1, 53.5, 36.3, 28.0, 20.0, 12.7, 7.4, and 1.4 MPa) were tested by adjusting the quantity of glycerol in the rehydration solution.

2.5. Measurement of yeast viability

Cell viability was estimated in triplicate by the CFU method. After osmotic treatments, fully rehydrated cells were serially diluted and the appropriate dilutions were plated onto three plates. CFU were counted after an incubation of 36 h at 25 °C. The initial cell suspension was used as the control.

2.6. Cell labelling

2.6.1. Cell labelling for flow cytometric assays

PI was used to assess membrane integrity because it can enter permeabilized cells and stain their DNA. BOX was used to evaluate changes in membrane potential. It can penetrate depolarized cells, where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence and a red spectral shift. Stock solutions of each dye were prepared as follows: PI was made up at 200 μg/mL in distilled water and BOX at 1 mg/mL in dimethyl sulfoxide. An aliquot (1 mL) of cell suspension (10^6 cells/mL) in a glycerol/phosphate buffer solution was labelled with 10 μL of PI and 3 μL of BOX stock solutions (final probe concentrations of 2 μg/mL PI and 3 μg/mL BOX) for 10 min in the presence of 4 mM EDTA to facilitate staining with BOX.

2.6.2. Cell labelling for confocal microscopy

The impermeant dyes PI and LY were used to evaluate membrane permeability and integrity. PI stains permeabilized cells, whereas LY enters cells by endocytosis [25,29]. PI and LY were dissolved in distilled water (10 mg/mL) to prepare the stock solutions. An aliquot (1 mL) of cell suspension (10^8 cells/mL) was washed twice with phosphate buffer/glycerol solution at 1.4 MPa and the supernatant was discarded. Phosphate buffer/glycerol solution (1 mL) containing 500 μg of LY and 200 μg of PI at the desired osmotic pressure was suddenly added to the pellet. After incubation at 25 °C for 30 min, the cells were washed twice with a phosphate buffer/glycerol solution at the same osmotic pressure without LY. The cells were observed by confocal microscopy immediately after the treatment for all conditions tested.

2.7. Confocal laser scanning microscopy

Confocal microscopy images were acquired at the Centre de Microscopie Appliquée à la Biologie (CMAB, Université de Bourgogne, Dijon, France). A Leica TCS SP2 confocal system attached to a research Leica DM RXA2 microscope (Leica Microsystèmes, Mannheim, Germany) was used with an argon/krypton air-cooled laser (488 nm) and a helium/neon laser (543 nm). Images were obtained with a ×63 oil-immersion objective (numerical aperture, 1.4). The Ar/Kr and HeNe lasers were used at 30% and 45% power, respectively, to minimize photobleaching.

2.8. Flow cytometry

Flow cytometric analyses were carried out at the "plateau technique de cytometry" (Université de Bourgogne, IFR 100, Dijon, France). The cell suspensions were analysed in a Coulter Epics Elite flow cytometer (Beckman Coulter France S.A., Paris, France) with 488 nm excitation by an argon-ion laser at 15 mW. Discriminators were set on forward (FSC) and side (SSC) scatter signals to reduce electronic and small-particle noise. The optical filters were set so that PI fluorescence was measured at 610 nm and BOX fluorescence at 525 nm. In all cases, there was some spectral overlap between the emitted fluorescence of the stain mixtures. Therefore, the compensation of the system was set to eliminate interference. Positively and negatively stained controls for each osmotic pressure were used to achieve compensation and exclude the contribution of signals from the yeasts. Typically, signals from 20,000 cells were acquired and analysed for each sample using the Window Multiple Document Interface for Flow Cytometry (Win MDI) 2.8 software. The flow cytometry results presented in this study are representative of at least three independent experiments.

3. Results

3.1. Yeast cells dehydration

3.1.1. Cell volume as a function of osmotic pressure (from 1.4 MPa to 103.9 MPa)

Fig. 1 shows the volume changes in the yeast cells from the physiological osmotic pressure (160 μm^3 at 1.4 MPa) with increasing levels of osmotic pressure ranging from 14.5 MPa to 103.9 MPa. For each osmotic pressure tested, the volume remained constant during for at least 1 h after the osmotic shock. The cell volume decreased exponentially from a small increase in osmotic pressure and reached 90 μm^3 at 14.5 MPa. At higher osmotic pressures, the volume decreases were smaller and reached a minimum value of 55–65 μm^3 at around 50 MPa, which corresponds to 40% of the initial volume.

3.1.2. Assessment of membrane functionality and integrity during exposure to hyperosmotic treatments in the range from 14.5 MPa to 70.3 MPa

Yeast cells were submitted to hyperosmotic shocks from 1.4 MPa to 14.5, 30.7, 49.1, or 70.3 MPa. The cells were then labelled with PI and BOX and analysed by flow cytometry after exposure to that hyperosmotic pressure for 10, 20, or 30 min to assess the functionality and integrity of their plasma membranes under such conditions. Three sub-populations of yeasts were distinguished: intact non-stained cells; cells with depolarized membranes, which were stained only with BOX; and BOX/PI double-stained cells, with permeabilized membranes. The maximal osmotic pressure that could be analysed by flow cytometry was 70.3 MPa because of the high viscosity of the glycerol solutions above this level. The changes in the percentage of cells with depolarized and permeabilized membranes as a function of osmotic pressure are presented in

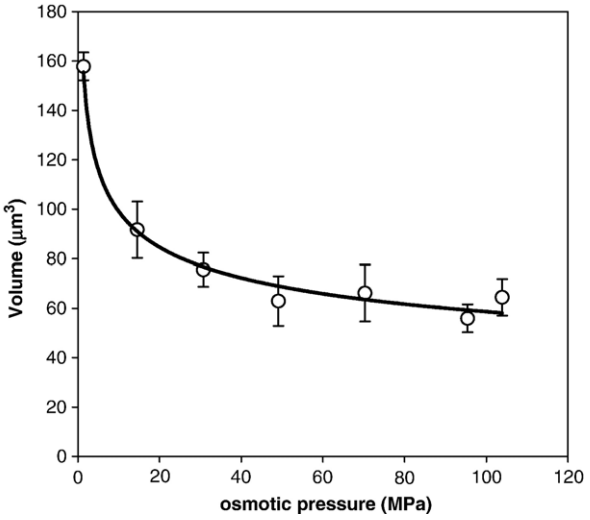

Fig. 1. Evaluation of the volumes of cells as a function of osmotic pressure in water/glycerol solutions. Cells initially equilibrated at 1.4 MPa in a glycerol solution were submitted to osmotic shocks at final osmotic pressures ranging from 14.5 Mpa to 109.9 MPa. Error bars correspond to confidence intervals at the 0.05 level.

Fig. 2A and B, respectively, for the three exposure times tested. Fig. 2A shows that no cells were depolarized by osmotic treatments at 14.5 MPa or 30.7 MPa. After 10 min under hyperosmotic conditions, 4.6% and 57.1% of cells were depolarized at 49.1 MPa and 70.3 MPa, respectively. At 49.1 MPa, depolarized cells reached 7.4% but returned to 3.7% after 30 min. At 70.3 MPa, the number of depolarized cells decreased during the exposure time from 57.1% to 34.6% and then to 27.6% after 20 and 30 min, respectively, indicating that a membrane polarization was restored in a proportion of the cells. The number of depolarized cells was then constant for more than 1 h (results not shown). Fig. 2B shows that the percentage of permeabilized cells was less than 1% after osmotic shocks at 14.5 MPa and 30.7 MPa. This percentage increased slightly during exposure to 49.1 MPa and 70.3 MPa. At 49.5 MPa, the percentage of permeabilized cells increased from 0.7% after 10 min, to 1.7% after 30 min. At 70.3 MPa, it increased to 3.3% after 10 min and 5.4% after 30 min. Thus, permeabilization remained a minor event in the whole cell population (<5.5%).

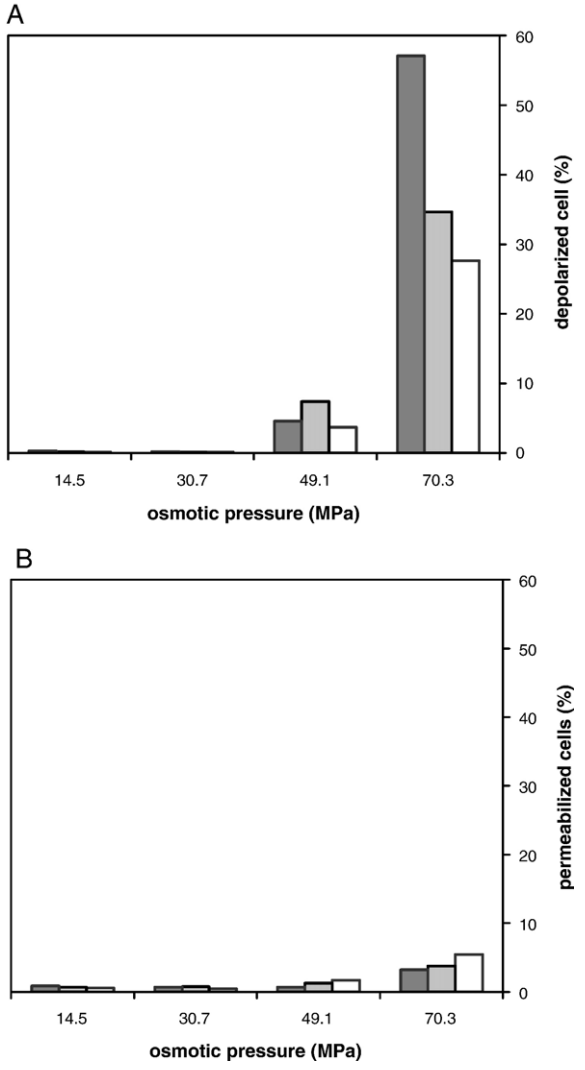

Fig. 2. Changes in the percentage of depolarized cells (positively stained with BOX) (A) and permeabilized cells (positively stained with PI and BOX) (B) measured by flow cytometry after hyperosmotic shocks from 1.4 MPa to four final osmotic pressures, for different maintenance periods under those hyperosmotic conditions : 10 (▩), 20 (▨), and 30 (□) min.

3.1.3. Assessment of membrane permeability during exposure to hyperosmotic treatments from 1.4 MPa to 144.5 MPa

Confocal microscopy was used to study the membrane status of yeasts after hyperosmotic shocks and under osmotic pressures of 17.6, 79.8, 109.9, and 144.5 MPa,. This allowed us to study changes in membrane integrity above 70.3 MPa, for which the flow cytometric technique could not be used. Osmotic shocks were applied in the presence of LY and PI and cells were observed after 30 min incubation at high levels of osmotic pressure. A representative example of the images acquired at 109.9 MPa is presented in Fig. 3. A differential scanning Normaski image (Fig. 3A), and laser scanning confocal images at 520–571 nm (LY emission) (Fig. 3B) and at 637–665 nm (PI emission) (Fig. 3C) are presented of the same field. The three acquired images were also superimposed (Fig. 3D). The Normaski image (Fig. 3A) shows that some cells appear granulous, with relief on their surfaces (black arrows), whereas other cells are smooth. In Fig. 4B, most of the cells are labelled with LY, but with different levels of fluorescence intensity. The outline of all the cells are also labelled with PI (Fig. 3C), whereas PI penetrated only seven of the 19 cells in the field shown. Cells labelled with PI show heterogeneous labelling, with brighter fluorescent patches at the centres of the cells. The superimposed images (Fig. 3D) show that all the cells with PI stained interiors are also stained with LY and have a granulous appearance when observed with transmitted light and a Normaski filter (arrow 1 in Fig. 3D). In some cells, the cytoplasm is only stained with LY (arrow 2 in Fig. 3D). Among these cells, some display bright LY fluorescence and other are less luminous (arrows 3 and 4 in Fig. 3B). Thus, confocal microscopy revealed the existence of three subpopulations of cells based on labelling: intact unstained cells; PI/LY double-stained cells with permeabilized membranes and LY-stained cells.

Cells were observed at four osmotic pressures (17.6, 79.8, 109.9, and 144.5 MPa), and at 1.4 MPa as the control. The labelled cells were counted at each osmotic pressure tested,. The results of counting 200 to 300 cells in two independent experiments per osmotic pressure are presented in Fig. 4. Whereas less than 1% of cells were labelled with PI in the control at 1.4 MPa, around 10% of cells were loaded with LY under physiological conditions. There was no difference between cells at 17.6 MPa and the control cells. The percentages of PI/LY double-stained cells and LY-stained cells increased from 14.6 MPa to 109.9 MPa, reaching 26.6% and 50.3% for permeabilized and LY-labelled cells, respectively. The percentage of permeabilized cells was approximately the same at 109.9 MPa (26.6%) and 144.5 MPa (23.7%). The percentage of LY-stained cells increased to 56.9% at 144.5 MPa. The number of LY-stained cells did not differ when LY was added before or just after an osmotic shock at 109.9 MPa (results not shown). This indicates that LY loading occurred during the incubation

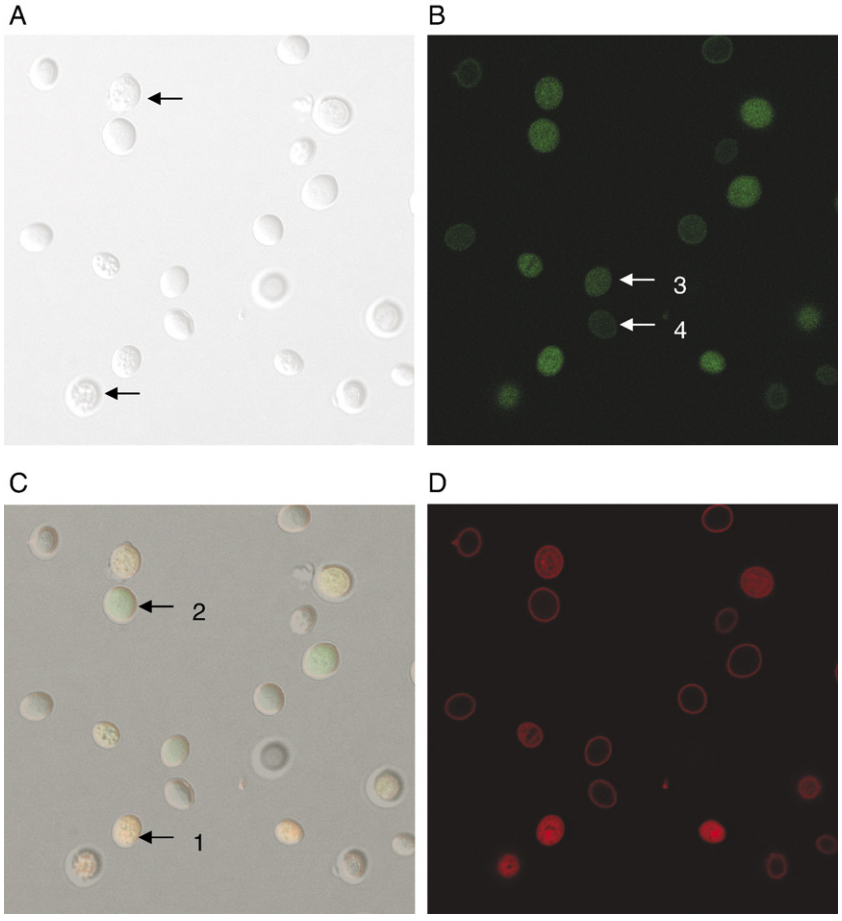

Fig. 3. Images of cells incubated for 30 min with PI and LY after an osmotic shock from 1.4 MPa to 109.9 MPa. The cells were washed twice with a water/glycerol solution at 109.9 MPa without dyes before their observation under hyperosmotic conditions by confocal microscopy (×63 oil-immersion objective). (A) Differential scanning Normaski image. (B) Laser scanning confocal image with 488 nm excitation/521–571 nm emission. (C) Laser scanning confocal image with 543 nm excitation/637–665 nm emission. (D) Superimposed images.

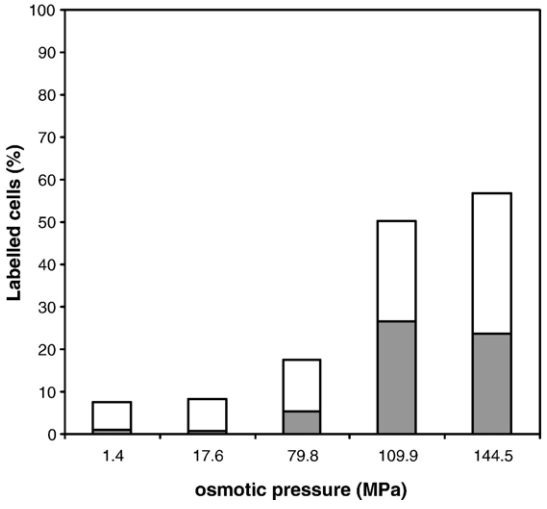

Fig. 4. Percentage of pemeabilized cells (PI/LY double-stained cells) (▨) and cells labelled only with LY (□) after hyperosmotic shocks from 1.4 MPa to four different final osmotic pressures and 30 min exposure to hyperosmotic conditions. At least 200 cells were counted in microscopy images for each osmotic pressure tested.

period under hyperosmotic pressure and not during the acute phase of the osmotic shock.

3.2. Yeast cell rehydration

3.2.1. Assessment of membrane functionality and integrity at different levels of rehydration from 72.1 MPa to 1.4 MPa after a hyperosmotic shock at 144.5 MPa

Plasma membrane functionality and integrity were assessed during the return of cells to the initial osmotic pressure. Cells previously shocked at 144.5 MPa and maintained for 30 min at that osmotic pressure were rehydrated by hypoosmotic shocks to 72.1, 53.5, 36.3, 28.0, 20.0, 12.7, 7.35, or 1.4 MPa. The cells were then labelled with PI and BOX and analysed by flow cytometry. The results are presented in Fig. 5. Three subpopulations of yeasts were distinguished: intact non-labelled cells; depolarized cells stained with BOX; and permeabilized cells double-stained with BOX and PI. For rehydration levels between 72.1 MPa and 20 MPa, the percentage of permeabilized cells was almost constant at 30–40%. The remaining cells were either depolarized or intact. The percentage of intact cells was maximal at 20 MPa (51.6%), whereas the percentage of depolarized cells decreased from 32.3% at 72.1 MPa to 11.6%

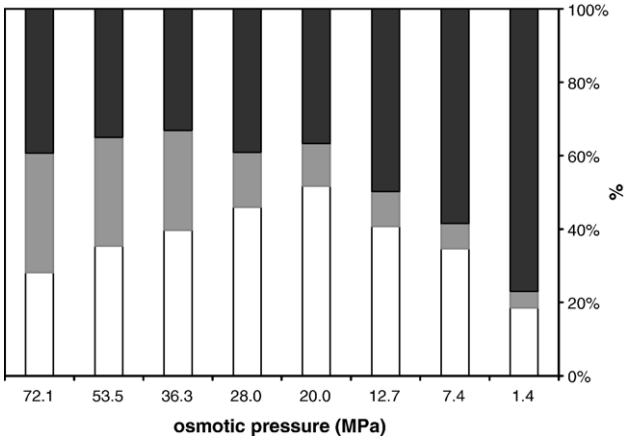

Fig. 5. PI and BOX staining of cells submitted to a hyperosmotic shock at 144.5 MPa followed by hyperosmotic exposure for 30 min and a return to a lower osmotic pressure, ranging from 72.1 MPa to 1.4 MPa, with an hypoosmotic shock, evaluated by cytometry. PI/BOX double-stained cells (■) have permeable membranes; BOX-stained cells (▩) have depolarized membranes; and unlabelled cells (□) have intact membranes.

at 20 MPa. At higher rehydration levels (osmotic pressure <20 MPa), the percentage of permeabilized cells increased from 36.6% at 20 MPa to 76.8% at 1.4 MPa. The proportion of depolarized cells decreased from 11.6% to 4.5% and the proportion of intact cells decreased from 51.7% to 18.5%.

3.2.2. Assessment of functionality, integrity and viability after different levels of dehydration and rehydration to 1.4 MPa

Cell viability was measured by the CFU method after hyperosmotic shocks of various intensities, maintenance for 30 min at that hyperosmotic pressure, and rehydration to 1.4 MPa. Fig. 6 shows that cell viability was not affected by osmotic shocks until 68 MPa. However, only 80.6% of cells were viable after an osmotic shock at 90 MPa and return to 1.4 MPa. Then viability decreased linearly with the magnitude of the osmotic shock, reaching 32.6% after an osmotic shock at 144.5 MPa. Flow cytometry analysis was conducted in parallel, to evaluate the permeability and functionality of the cell membranes after the same treatments at three osmotic pressures. Fig. 7 presents the PI and BOX staining of cells after osmotic shocks at 49.1, 95.4, or 144.5 MPa and rehydration to 1.4 MPa. Three subpopulations of yeasts were distinguishable. The proportions of intact cells were 96%, 72% and 26% after hyperosmotic shocks at 39.6 MPa, 95.4 MPa, and 144.5 MPa, respectively. These values correspond to the viability percentages measured by the CFU method after identical hyperosmotic shocks. After hyperosmotic shocks of 39.6, 95.4 and 144.5 MPa, 100%, 80.6%, and 32.6% of cells replicated (see Fig. 6).

4. Discussion

In this study, the functionality and integrity of the yeast plasma membrane were assessed at the two stages of an osmotic treatment : dehydration and rehydration. A sequence of the events that occur with increasing levels of dehydration and then during rehydration is proposed below.

4.1. Sequence of the membrane events during the dehydration step: response of cells to increasing hyperosmotic shocks

4.1.1. 1.4 MPa to 70.3 MPa: decrease in cell volume and subsequent membrane depolarization

Sudden exposure to a hyperosmotic stress causes rapid equilibration of the osmotic pressures of the cytoplasm and the external medium. During the transitional step of the passive osmotic response, water flows out of the cells, leading to cell shrinkage [11,30,31] and permeant solutes, such as glycerol, penetrate into the cells. This exchange is very fast [8] and ends up in a stationary step, when osmotic pressures are equilibrated. In this study, cell volume decreased exponentially between 1.4 MPa and 49.1 MPa, before reaching a constant volume corresponding to 40% of the volume of the fully hydrated cells at higher osmotic pressures (Fig. 1). Cell volume was evaluated from light microscopy images and thus took into account the total envelope of the yeast, i.e., cell wall and membrane. In contrast to plant cells and bacteria, in which the plasma membrane shrinks away from the cell wall, the entire cell volume shrinks when yeast cells are placed in hypertonic solutions [10,32]. Considering the poor compressibility of biological membranes [33], this strong cell shrinkage, must be associated to wrinkling of the membrane. Adya et al. [9] reported a decrease of about 50% in the volume of *S. cerevisiae* subjected to an osmotic stress in 40% (w/v) sorbitol and an increase in surface roughness with increasing exposure times. According to those authors, the cell shape became irregular. Such changes in cell shape could not have been observed in our study.

However, the minimum volume reached during dehydration corresponded to the appearance of the first depolarized cells at 49.1 MPa. After shock and exposure to 70.3 MPa, when the observed volume contraction was maximal, the number of depolarized cells increased dramatically (Fig. 2A). The polarization of biological membranes results from a balance between pump-based transport and channel-based leakage. The most important ions implicated in gradients across bio-

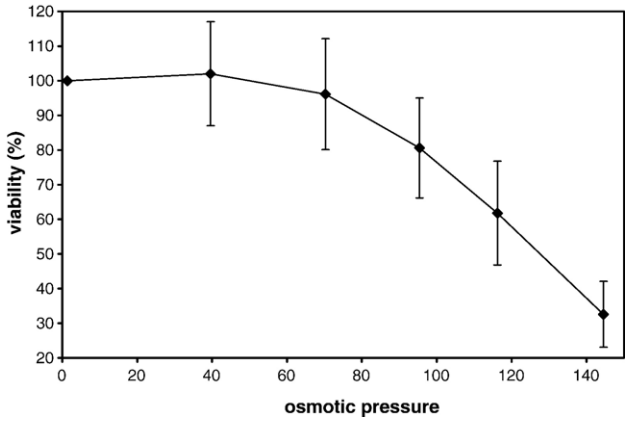

Fig. 6. Percentage viability of yeast cells after hyperosmotic shocks of various magnitudes followed by a 30-min exposure to that osmotic pressure. Viability was measured by the CFU method after return to 1.4 MPa in a hypoosmotic shock. Error bars correspond to confidence intervals at the 0.05 level.

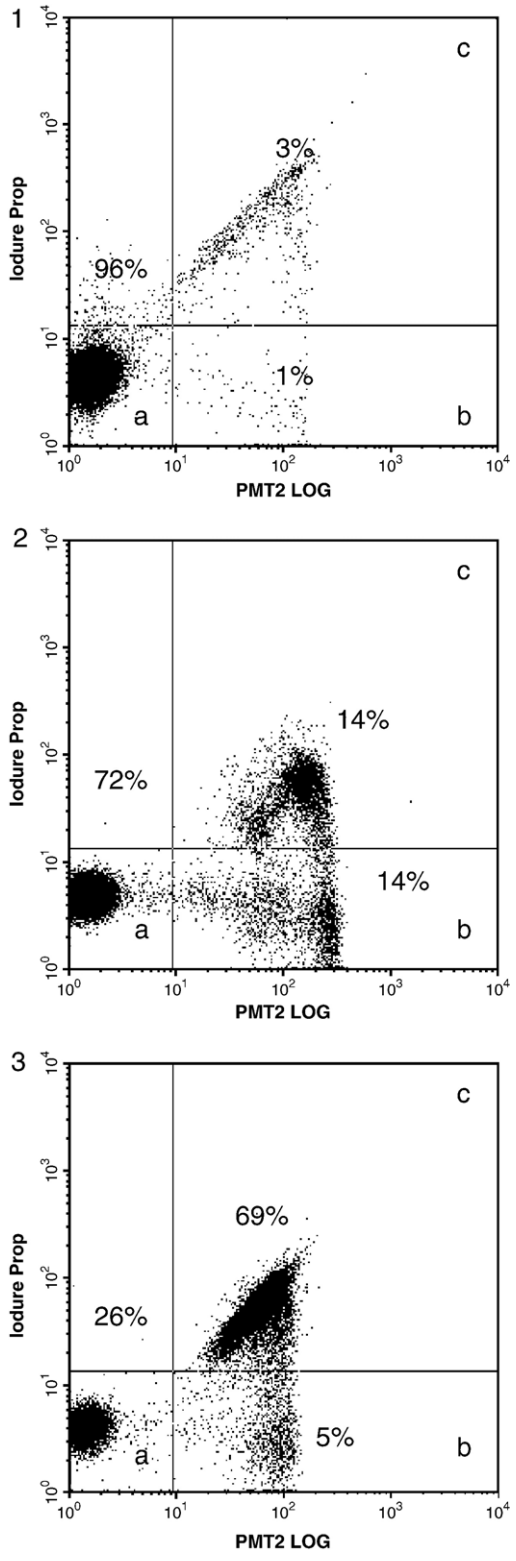

Fig. 7. Cell samples (20, 000 cells) analysed by flow cytometry after hyperosmotic shocks at 39.6 MPa (1), 95.4 MPa (2), and 144.5 MPa (3), followed by a hyperosmotic exposure for 30 min, and return to 1.4 MPa. Three main subpopulations of cells correspond to healthy polarized cells without staining (a); cells with no membrane potential, stained with BOX (b); and cells with permeabilized membranes, stained with PI and BOX (c).

membranes include Na^+, K^+, H^+, Ca^{2+} and Cl^-. This energy-requiring disequilibrium is maintained by the action of specific electroneutral pumps (e.g., Na^+/H^+ symports), channels (e.g., those that facilitate K^+ and Ca^{2+} transport), and electrogenic pumps (H^+-translocating ATPases). Because of its complexity, the mechanism that determines the homeostasis of intracellular ions in yeast remains largely uncharacterized [34]. The depolarization of cells following a hyperosmotic shock at 70.3 MPa may result from the consequent release of ions into the extracellular medium, as has already been observed for K^+ [12], or from a decrease in the intracellular pH due to an increase in the proton concentration, as a consequence of the contraction of the cell volume, as shown by Vindelov and Arneborg [35].

Another interesting observation is related to the percentage change in the number of depolarized cells during the period of exposure to hyperosmotic conditions at 70.3 MPa indicating that the polarization of the plasma membranes of the cells was restored at this osmotic pressure. In fact, 30% of the cells recovered their polarized status after 10–30 min exposure at 70.3 MPa (see Fig. 2A). It is well known that mechanosensitive ion channels are activated when the membrane is stretched and play a role in the regulation of ion homeostasis [36]. The activation of mechanosensitive ions channels in response to a hyperosmotic stress induced with sorbitol has already been observed in *S. cerevisiae* at 4 MPa [37]. Now, transferring yeast cells to a sorbitol solution at this osmotic pressure provokes a decrease in the cell volume that reaches 65% of the initial volume [31]. Therefore, regarding the magnitude of cell shrinkage, our hypothesis is consistent with these results and, in our experiments, mechanosensitive ion channels may have been functional in a proportion of the cells. It would permit the change of ionic conductance of the plasma membrane and their passive repolarization. This hypothesis needs to be investigated. However, 27.6% of cells remained depolarized after 30 min at 70.3 MPa.

Thus the sequence of cell changes after increasing hyperosmotic shocks could have initially involved cell volume contractions from a high osmotic pressure level (14.5 MPa) to 50 MPa (Fig. 1). Then, the plasma membrane was depolarized at 70.3 MPa (57%) but at least 30% of cells became repolarized during the 30 min exposure to 70.3 MPa (Fig. 2A). Moreover, according to Fig. 6, the depolarization of the plasma membrane and cell contraction were not related to cell death because cell viability measured after rehydration was 96% for an osmotic shock at 70.3 MPa , with exposure for 30 min. However, this is the limit beyond which cells began to die. In fact, 10% of cells died after an osmotic shock at 95.4 MPa (Fig. 6).

4.1.2. 79.8 MPa to 144.5 MPa: plasma membrane permeabilization to PI and LY and release of cell components

Changes in plasma membrane permeability have usually been considered the main cause of cell death during dehydration [18,19]. Actually, our results show that the number of PI-stained cells (permeabilized cells) increased with increasing osmotic pressure from 70.3 MPa to 109.9 MPa (Figs. 2B and 4). Therefore, this osmotic pressure interval appears to be critical

for membrane permeability during dehydration. Phase transitions of phospholipids have been proposed as the cause of the increase in membrane permeability in both phospholipid vesicles [38] and yeasts [39] under osmotic stress. Water loss from phospholipid head groups may lead to phase transitions in some lipids, resulting in a lateral phase separation [40,41], which allows the leakage of intracellular contents [17]. The occurrence of a phase transition in yeast membrane lipids from 61.8 MPa to 133.1 MPa in glycerol solution at an average temperature of 22 °C [39] could explain the permeabilization detected in this study between 70.3 MPa and 109.9 MPa.

During hyperosmotic treatments, the number of LY-stained cells also increased with increasing osmotic pressures to final levels ranging from 79.8 MPa to 144.5 MPa (Fig. 4). LY is a membrane-impermeant anionic dye. This polar tracer is usually loaded by microinjection, pinocytosis, or scrape loading. It has been used to characterize endocytosis in plant cells [24] and yeasts [25] in which the presence of a cell wall prevents the access of high-molecular-weight molecules to the plasma membrane. The fact that the same number of cells was stained with LY when the dye was added before or just after the osmotic shock indicates that LY penetrated into the cell interior during incubation under hyperosmotic conditions. In PI/LY double-stained cells, LY probably penetrated into the cells because the plasma membranes were permeabilized. In cells stained only with LY, two hypotheses can explain the entry of LY into the cell interior. The first hypothesis is the existence of two levels of plasma membrane permeabilization, defined by the penetration into the cell of only LY or of both PI and LY. A second hypothesis is the occurrence of plasma membrane endocytic vesiculation under hyperosmotic conditions, as already observed by Mille et al. [42] with *Escherichia coli* and by Okada and Rechsteiner [43] in mammalian cells. Slaninova et al. [32] reported the occurrence of deep plasma membrane invaginations filled from the periplasmic side with an amorphous cell wall material, when *S. cerevisiae* cells were transferred to hyperosmotic growth medium. Such invaginations, when associated with lipid phase separation induced by dehydration, could lead to the formation of endocytic vesicles. In fact, Liu et al. [44] recently showed that the scission of membrane invaginations could be promoted by lipid phase separation to form endovesicles. We did not observe LY-stained endocytic vesicles in the cell interior, but the rapid accumulation of LY in the vacuole could explain this [25]. In our experiments, several levels of fluorescence intensity were distinguishable in the LY-stained cells. This observation may reflect differences in the quantities of LY internalized by different cells after incubation for 30 min. The extent of the phenomenon is probably time-dependent and may occur as long as an excess of membrane surface exists regarding the volume of the cells.

At 109.9 MPa and 144.5 MPa, outline of the cells were labelled with PI under hyperosmotic conditions, indicating that PI-stained material, probably RNA or small nucleotide fragments, was present around the cells. Dehydrated cells release various substances, including ions, nucleotides, amino acids, lipids, peptides, and proteins [12]. A transitory change in membrane permeability may have occurred during the acute hyperosmotic shock period, i.e., during water outflow and cell volume contraction, which may have provoked macromolecules release without permitting PI or LY to penetrate into the cell interior. The strong contraction of the cell wall would then have prevented the outflow of macromolecules from the periplasmic space. Isolated walls are only permeable to molecules of molecular masses up to 760 Da. [45]. Because the molecular mass of a nucleotide ranges between 300 and 500 Da, an oligonucleotide ejected from the cell interior would be unable to cross a contracted cell wall.

4.2. Sequence of membrane events during the rehydration step: response of cells to increasing rehydration shocks

4.2.1. Increasing rehydration levels from 70.3 MPa to 1.4 MPa after dehydration at 144.5 MPa: existence of a rehydration level with a maximum survival rate

The changes in the proportions of the three subpopulations of yeast cells as a function of the level of rehydration (Fig. 5) after a hyperosmotic shock at 144.5 MPa show that the percentage of BOX-stained cells decreased for osmotic pressures of rehydration ranging from 72.1 MPa to 20 MPa, with a concomitant increase in intact cells. Therefore, plasma membrane depolarization was reversible in this osmotic pressure interval, and in this study, yeast plasma membrane integrity and functionality were best maintained at a level of rehydration of 20 MPa. It is likely that cell depolarization was counteracted more and more efficiently as the hydration level increased. Therefore, active ion transporters may have been functional. As mentioned above, ion influx occurs under hyperosmotic stress [37]. However it has never been observed at such a high osmotic pressure. The percentage of permeabilized cells (PI-stained cells) was constant between 70.3 MPa and 20 MPa during rehydration and increased strongly at the upper levels of rehydration, showing that most of the cells, that had reached a critical osmotic pressure (144.5 MPa) could not recover their initial osmotic pressure (1.4 MPa). Therefore, the osmotic pressure interval between 20 Mpa and 1.4 MPa appears to be critical for membrane permeability during rehydration. The existence of this critical step could be related to membrane events that occur during dehydration. Indeed, after dehydration at 144.5 MPa, cells stained only with LY represented 35% of the cells (Fig. 4). These may have suffered from a reduction in surface area associated with the formation of endovesicles, as has already been proposed by Shalaev and Steponkus [46] and is supported by our observations. Therefore, exposing these cells to rehydration levels that impose significant increases in volume, i.e., above 20 MPa (Fig. 1), may result in their lysis during volume expansion. Okada and Rechsteiner [43] reported that endovesicles that form under hyperosmotic conditions swell and burst upon rehydration of the cytosol. Fig. 8 schematizes the sequence of events observed at different levels of the rehydration step following hyperosmotic shock at 144.5 MPa. It shows that the proportion of pemeabilized cells between 20 and 1.4 MPa (40%) is close to the proportion of cells stained with only LY at 144.5 MPa (35%), which reinforces the previous hypothesis.

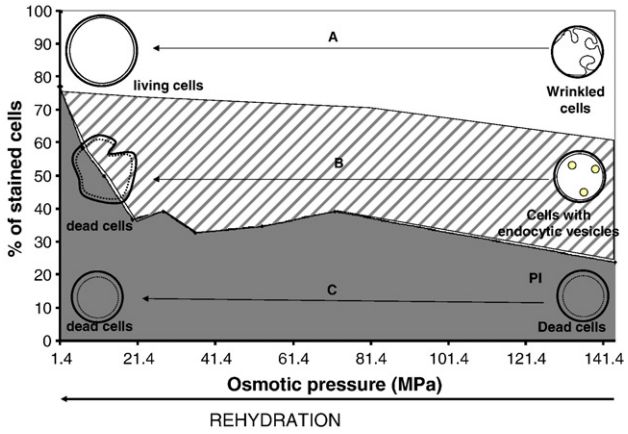

Fig. 8. Schema of the hypothetical sequence of events occurring during cell rehydration from 144.5 MPa to 1.4 MPa. The proportions of propidium iodide (PI)-stained cells (▩) represent experimental values, whereas the proportions of cells with endocytic vesicles (▨) are extrapolated from data obtained at 144.5 MPa. Intact non-stained cells (□) recovered their initial volumes (A). Cells with endocytic vesicles were permeabilized when they were rehydrated to osmotic pressures lower than 20 MPa, at which cell volumes increased again (B). Some cells were permeabilized during the dehydration step (C).

4.3. Rehydration to 1.4 MPa after exposure of yeast cells to different levels of dehydration

The CFU method was used to evaluate yeast viability after osmotic treatments (hyperosmotic shocks and return to 1.4 MPa) of various magnitudes. Cells viability was not affected by osmotic shock until 70.3 MPa, at which osmotic pressure only reversible membrane depolarization was observed. With stronger osmotic shocks, cells crossed a critical osmotic pressure range for membranes during dehydration (70.3 Mpa to 109.9 MPa, Fig. 4) or rehydration. The decrease in viability was proportional to the increase in osmotic pressure, confirming the strong involvement of the plasma membrane in the mechanisms leading to cell death during osmotic treatments.

CFU-measured cell viability was compared with the PI and BOX staining of cells after the same treatments. The percentage of cells able to replicate corresponded to the percentage of intact cells, which lacked staining (Figs. 5 and 6). Thus, BOX-stained cells were alive, but unable to replicate. Our results are in agreement with those of other studies in which BOX was used to evaluate the "vitality" of cells [47] wherein BOX staining correlated well with the loss of yeast fermentation activity [14]. Thus, PI/BOX double staining after rehydration allows us to distinguish vigorous, injured, and dead cells.

4.4. When do cells die during osmotic treatment?: a hypothetical mechanism leading to cell death

Our study results allow us to propose a mechanism leading to cell death during the dehydration–rehydration process. We have been focused on membrane physical changes during the treatments to propose this mechanism. We dismissed a hypothesis of a possible toxic effect of glycerol entry into cells. In fact, glycerol is the main synthesised osmolyte in yeast [11]. Moreover, this is a compatible solute, meaning that it is a neutral substance expected not to be toxic for enzymes in the cytoplasm [48] and the quantity of glycerol a yeast cell can accumulate is very high [49,50]. For example, Wojda et al. [51] report an intracellular glycerol concentration of 900 μg/mg protein in a wild-type strain of *S. cerevisiae.* In addition, the fact that glycerol provokes osmotic stress for yeasts has been proven many years ago [52]. In one of our team's earlier study [53], 90% of yeast survival could be obtained after an osmotic treatment at 100 MPa in a glycerol solution by increasing slowly the osmotic pressure. Therefore, it is improbable that glycerol is toxic for yeasts and as far as we know, such a toxic effect has not been reported in the literature.

For osmotic shocks higher than 70.3 MPa, the removal of a portion of water from the cells may lead to changes in the permeability of the cells resulting from the phase separation of phospholipids, which may occur in the range of osmotic pressures of 61.8–133.1 MPa according to Laroche et al. [39]. In fact, lipid phase transition affects the resistance of membranes to shear forces [54,55] and volume contraction may thus be critical when this occur. Therefore, a proportion of cells die because their membranes become permeabilized (PI-stained cells) during the dehydration stage. Other cells may undergo a reduction in the surface area of their plasma membranes by endocytic vesiculation. When these cells are rehydrated, the proportion of permeabilized cells is stable for rehydration levels that do not induce important volume increases. If rehydration involves a significant volume increase (rehydration to osmotic pressures <20 MPa according to Fig. 1), the reduction in area of the plasma membrane causes cell lysis during osmotic expansion. Thus, there is a rehydration level, at 20 MPa, at which the percentage of cells with intact membranes is maximal.

To conclude, plasma membrane changes are strongly implicated in the mechanism leading to cell death during osmotic dehydration and rehydration. In particular, permeabilization resulting from lipidic phase transitions and severe volume contractions could explain the observed sequence of events. Moreover, the changes that occur during the dehydration step and the rehydration step are interdependent. This study increases our knowledge of the mechanism leading to cell death during the osmotic dehydration–rehydration process. It emphasizes the need to consider each step of an osmotic treatment in optimizing survival of yeasts exposed to such environmental stresses.

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